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Validation of GUt Low Density Array (GULDA), a novel qPCR approach to the study of the intestinal microbial ecosystem

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Introduction:

Causal relationships between the vast numbers of bacterial species present in the human intestines contain a lot of potential information on the regulation of the gut in the healthy as well as in diseased states. Based on the hypothesis that the human gut microbiota constitutes a dynamic ecosystem, interesting correlations between the presences of the given species should exist at any time. In order to analyse this, we have developed GULDA, a cheap, flexible, reliable and high throughput qPCR-based gut low-density array (GULDA), which simultaneously gives the quantities of a selected number of bacterial 16S rRNA targets on all relevant phylogenetic levels in a given sample of DNA. In comparison to other strategies e.g. metagenomic sequencing and microarrays, GULDA focuses on selected targets only and requires only little complex bioinformactical post-processing.
Given the setup, where one standard qPCR program is used for 42 primer sets, validation is important. We present here strategies involved in verification of GULDA as a valid tool for analysis of the human gut microbiota.

Fig 1: GULDA qPCR setup

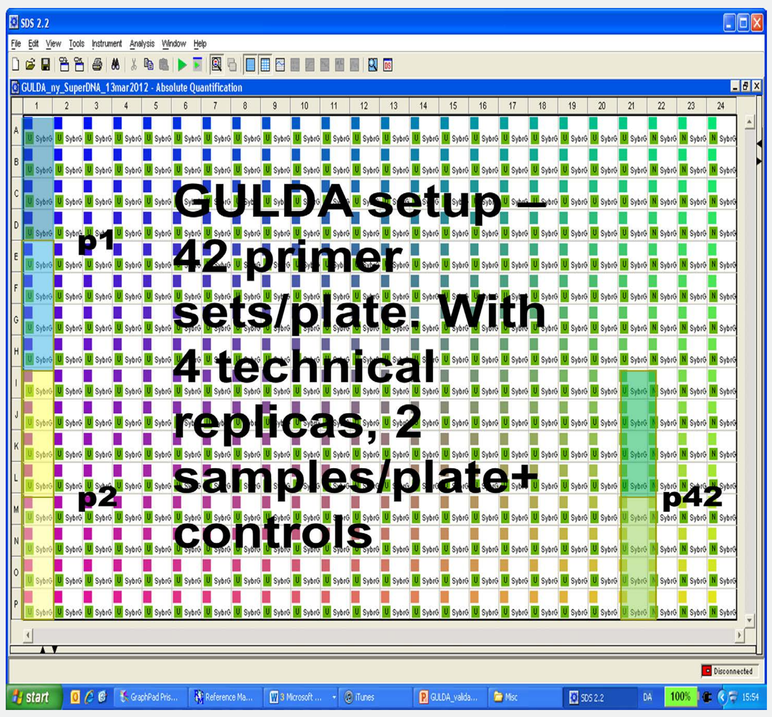

Fig 1: 16S rRNA targeting primers (p1,p2, …p42) run on the same qPCR plate in 4 technical replicas for each sample. Template-free controls run on the far right of each plate to ensure specificity. The GULDA-setup is unique as it allows the concomitant analysis of a very diverse set of bacterial targets as all included primer sets were optimized to run under the same PCR conditions i.e. same PCR program. Only bacterial targets of interest are analyzed, while individual 16S rRNA template genes can be added or removed easily. In details:
Quantitative real-time PCR was performed on the ABI prism 7900HT from Applied Biosystems. The amplification reactions were all carried out in 384-well MicroAmp® Optical 384-well reaction plates (Applied Biosystems, Nærum, Denmark) and sealed with MicroAmp® Optical Adhesive Film (Applied Biosystems, Nærum, Denmark) in a total volume of 11µl containing 5.5 µl master mix (2x SYBR Green PCR Master Mix from Applied Biosystems, Nærum, Denmark), 0.4µl of each primer (10 µM), 2µl template DNA, and 2.7µl nuclease-free water (Qiagen GmbH, Germany) purified for PCR. Liquid handling was performed with the epMotion 5075 (Eppendorf, Hørsholm, Denmark). The amplification program consisted of one cycle of 50 C for 2 min; one cycle of 95 C for 10 min; 40 cycles of 95 C for 15s and 60 C for 1 min; and finally one dissociation curve analysis for assessing amplicon specificity (95 C for 15s, 60 C for 20s and increasing ramp rate by 2% until 95 C for 15s (until detection for each amplicon)).

Fig 2: Primer validation with bacterial culture DNA

Difference between the Ct-value recorded by qPCR on pure culture DNA for specific primers and for a universal bacterial primer targeting all bacterial 16S rRNA genes

Primers	Firmicutes phylum	Lactobacillus spp.	Lactobacillus plantarum	Lactobacillus acidophilus	Clostridium cluster I	Clostridium cluster IV	Clostridium cluster XIV	Faecalibacterium prausnitzii	Clostridium cluster XIV	Clostridium coccoides group	Eubacterium hallii	Roseburia spp.	Enterococcus spp.	Bacteroidetes phylum	Bacteroides/Prevotella group	Bacteroides spp.	Bacteroides fragilis group	Bacteroides vulgatus	Bacteroides thetaiotaomicron	Parabacteroides distasonis	Bacteroides eggerthii	Prevotella spp.	Alistipes spp.	Bifidobacterium spp.	Bifidobacterium bifidum	Bifidobacterium adolescentis	Bifidobacterium catenulatum group	Bifidobacterium longum	Bifidobacterium breve	Enterobacteriaceae	Escherichia coli	Desulfovibrio spp.	Akkermansia muciniphila	Methanobrevibacter smithii	Universal bacteria
Strains	p112	p32	p22	p30	p138	p60	p77	p69	p73	p72	p126	p84	p114	p128	p132	p119	p42	p118	p122	p146	p148	p131	140	p1	p4	p6	p156	p157	p134	p106	p103	p105	p142	p116
Lactobacillus acidophilus	1	2	17	2	17	17	17	17	17	17	17	17	16	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	18
Lactobacillus gasseri	-1	1	16	16	16	16	16	16	16	16	16	12	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	15	16	16	16	16	19
Lactobacillus plantarum	11	-1		23	19	23	23	23	23	23	23	16	17	21	23	23	23	23	23	18	21	23	21	23	23	23	23	23	22	23	23	23	23	12
Clostridium butyricum	0	26	26	26	1	26	26	23	25	26	26	26	26	26	22	26	7	26	26	26	26	26	26	26	26	26	26	26	26	26	24	26	26	9
Ruminococcus albus	0	22	22	22	22	0	22	22	22	22	22	22	19	22	22	22	22	22	22	22	21	22	13	22	9	22	22	22	22	22	12	22	22	13
Faecalibacterium prausnitzii	0	25	25	25	25	0	11	18	25	25	25	25	23	23	25	25	25	25	25	25	25	25	25	25	25	25	25	25	22	25	25	25	25	10
Blautia coccoides	0	14	19	19	3	19	19	0	2	19	19	19	11	16	17	19	19	19	19	17	14	19	19	19	19	19	19	19	17	19	12	15	17	16
Clostridium clostridioforme	0	20	23	23	12	15	23	1	2	23	23	18	12	14	14	16	23	23	23	22	23	23	23	23	23	23	23	23	17	23	9	21	22	12
Eubacterium Hallii	0	26	26	26	12	26	26	0	2	1	26	24	23	26	26	26	26	26	26	26	26	26	26	26	26	26	26	26	26	26	18	26	26	9
Roseburia faecis	0	22	22	22	22	21	22	3	22	22	0	22	14	14	17	15	14	22	22	22	22	22	22	22	22	22	22	22	22	22	15	22	22	13
Enterococcus faecalis	1	21	21	21	21	21	21	21	21	21	21	0	21	21	21	21	21	21	21	21	21	21	21	21	21	21	21	21	21	21	12	21	21	14
Bacteroides fragilis	23	23		23	23	23	23	23	23	23	23	23	0	-1	1	1	23	23	23	21	22	23	23	23	23	23	23	23	23	23	23	21	23	12
Bacteroides vulgatus	24	25	25	25	25	25	25	25	25	23	25	25	0	0	0	0	0	25	25	25	24	25	25	25	25	25	25	25	24	25	25	25	25	10
Bacteroides thetaiotaomicron	12	23	23	23	23	23	23	23	23	22	23	17	1	1	1	1	16	3	23	17	17	23	23	23	23	22	23	23	21	22	22	23	23	12
Parabacteroides distasonis	10	24	24	24	24	24	24	24	24	24	24	24	0	1	1	14	24	24	0	20	23	24	24	24	24	24	24	24	23	24	20	24	24	11
Bacteroides eggerthii	17	22	22	22	22	22	22	22	22	21	20	22	0	0	0	0	18	22	22	3	15	22	22	22	19	22	22	22	22	22	22	22	22	13
Prevotella copri	15	18	20	20	20	20	20	20	20	20	20	20	0	0	11	18	18	20	20	20	3	20	20	20	20	20	20	20	19	20	20	20	20	15
Alistipes shahii.	14	21	21	21	21	21	21	21	21	21	21	21	0	18	21	21	21	21	21	21	20	1	21	21	21	16	21	21	20	21	21	21	21	14
Bifidobacterium bifidum	3	20	20	20	20	20	20	20	10	20	20		17	17	19	20	20	20	20	20	20	20	1	1	20	20	20	20	20	20	20	20	20	15
Bifidobacterium adolescentis	2	19	19	19	19	19	19	19	19	19	19	19	19	19	19	19	19	19	19	19	19	19	1	19	0	19	19	19	17	19	19	19	19	16
Bifidobacterium pseudocatenulatum	4	21		23	23	23	23	23	23	23	23	20	19	23	23	23	23	23	23	21	22	23	2	23	23	2	23	23	23	23	22	23	23	12
Bifidobacterium longum	2	19		20	20	20	20	20	20	20	20	20	16	18	20	20	17	20	20	20	17	20	2	20	20	20	0	20	20	20	20	20	20	15
Bifidobacterium breve	4	19		19	19	19	19	19	19	19	19	19	19	19	19	19	19	19	19	19	18	19	2	19	19	19	19	3	19	19	19	19	19	16
Escherichia coli	13	23	23	23	23	23	23	23	23	23	23	21	15	21	16	23	23	23	23	17	19	22	23	23	23	23	23	23	0	2	23	22	23	12
Desulfovibrio vulgaris	15	25	25	25	25	25	25	25	25	25	25	25	18	25	20	25	25	25	25	25	22	25	19	25	25	25	25	25	25	25	0	25	25	10
Akkermansia muciniphila	15	24	24	24	24	24	24	24	24	24	24	24	21	23	22	24	24	24	24	21	23	24	24	24	21	24	24	24	22	24	24	0	24	11
Methanobrevibacter smithii smithii	9	19	20	20	20	20	20	20	20	20	20	18	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	0	20

Fig 2:
Columns: Primers on GULDA (total number less than 42 as the process is still ongoing.) Primers are organized by phylum. (The primer numbers are for internal use.)
Rows: Pure culture DNA representing all primers included. Strains organized by phylum.

All combinations of primers and DNA run optimized to run efficiently at the same PCR program (see GULDA setup to the left).
The raw Ct-value for the universal primer targeting all bacteria (p116) is in the last column. All other values, except M.smithii, was calculated as $\delta = C_t(P116) - C_t(Px)$, where Px denotes all other primers used. Color coding from green via yellow to red is proportional to δ. It is seen that for most primers, there is a very high level of specificity (green on the diagnoal), although the Firmicutes primer (p112) seem to amplify Bifidobacteria unspecifically. M.smithii is an archeabacteria, hence p116 is not a valid target, hence for the last row the approximation $\delta = C_t(P142) - C_t(Px)$, where p142 is M.smithii primer set, was used.

Additional validation and strategies

- All amplicons were run post-PCR on 2% agarose gel to verify the correct size of the qPCR product.
- Only primers giving one sharp dissociation curve in the validation test above were accepted.
- Amplicon-specific PCR efficiencies were calculated in order to take this potential bias into account as all primers run under the same qPCR conditions. Primers with highly (>10% from median) diverging efficiencies were excluded from further analysis.
- SybrGreen was preferred to probe based detection due to the high similarity in the 16S rRNA gene used as target. Probes could thus exclude amplification of interest.
- Amplicons have been isolated for 454-sequencing, but this has been postponed until further notice.
- The GULDA setup is to be tested on a number of fecal DNA samples in order to verify the strength of the tool for fast metagenomic analysis of fecal DNA samples (process ongoing)

Discussion

We have developed GULDA as a powerful metagenomic tool for rapid, reliable, flexible analysis of the human gut microbiota. Compared to already available metagenomic methodologies, GULDA is unique as it makes it possible to analyze a large number of bacterial targets simultaneously, without the concomitant detection and thus handling of enormous amounts of superfluous or irrelevant data with potential introduction of experimental bias. Moreover, the subsequent data analysis is straightforward and requires no advanced competences within bioinformatics. GULDA is useful, when comparing microbiota patterns between different kinds of individuals e.g. breastfed vs formula fed infants. It is the idea to use uni- as well as multivariate statistics e.g. Principal Component Analysis in the data analysis of associated microbiota patterns and developments. This should hopefully add to the knowledge of the intrinsic regulation of intestinal bacteria.

It is important to realize that it is not the purpose of GULDA to provide a method to quantify the relative levels of individual bacterial targets in a DNA sample. This would require exact knowledge on specific number of 16S rRNA gene copy number for each target, identical amplicon length (SybrGreen binds proportionally to the amplicon length) and highly similar composition and secondary structure of amplicons for comparison. It is obvious that such an analysis would be highly complicated, if at all possible.

The high level of specificity of all included primers shown here in combination with a validated approach to take into account the differences in PCR efficiency between the different amplicons, shows that GULDA provides a reliable method in the metagenomic analysis of the human gut microbiota.

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